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Final Progress Report
Assistance Award #DAMD17-99-1-9287
Role of Diet in the Initiation and Prevention of Breast Cancer

The purpose of the above titled postdoctoral traineeship was to complete several tasks focused upon further establishing the role of EST in the bioactivation of the cooked-food mutagen and breast carcinogen N-OH-PhIP and establishing how bioflavonoids may be used to prevent this bioactivation process. Task #1, as outlined in the statement of work, listed several goals to help define the involvement of EST in bioactivation of N-OH-PhIP in the normal human breast cell and tissue. Part of this task, characterizing the expression and localization of EST in normal human breast cells and tissue, has opened many new research opportunities. Although EST had previously been detected in cultured human mammary epithelial cells, EST had never been detected or its localization determined in normal human breast tissue. However, by using immunohistochemistry we have for the first time localized EST to the epithelial cells lining the ducts and alveoli of normal human breast tissue. This finding is not only novel but is interesting in that these very ductal epithelial cells are believed to be the site for tumor initiation by various carcinogens, including the breast carcinogen N-OH-PhIP.

By using a very sensitive technique, immuno-staining with confocal microscopy, we have also determined the subcellular localization of EST in cultured human mammary epithelial cells. We found EST present in the cytosol, as previously described by western blot analysis, but of great excitement was our new finding that EST, maybe even a novel isoform, is localized to the nuclei of these breast cells. The potential biological role of this nuclear EST remains to be determined.

Because of the importance of EST in bioactivation and in estrogen hormone regulation, we felt that the EST localization studies and the potential biological relevance of nuclear and cytoplasmic EST to breast cancer development must be further investigated. Therefore, we have submitted a *DOD concept grant application* and a *DOD idea grant*

application in hopes of obtaining more research funding to continue these studies.

We must mention that in addition to studying the EST localization proposed in Task #1, we also further explored bioactivation of N-OH-PhIP by EST. These results were published in *Carcinogenesis* (article previously submitted). In this study we established that EST expression increases with subsequent days following cell seeding, but we have yet to determine how EST expression effects DNA binding of N-OH-PhIP in normal breast cells.

Task #2, outlined in the statement of work, listed several goals to help define the inhibitory role of bioflavonoids on EST activity and EST-mediated binding of cooked-food mutagens to DNA. We have accomplished several goals listed under this task and our findings are currently in press in *The Journal of Steroid Biochemistry and Molecular Biology* (article enclosed). We have established the inhibitory effect of the dietary polyphenols quercetin and resveratrol on EST activity, i.e. 17 β estradiol. Both compounds potently inhibited recombinant human EST in a competitve fashion with Ki values of about 1 μM. In fact, both polyphenols could serve as substrates for EST. In order to extend the studies we examined whether inhibition of EST also occurred in the intact cultured human mammary epithelial cells. The IC_{50} for resveratrol was very similar to that for recombinant EST, i.e. about 1 μM. Surprisingly, quercetin was 10 times more potent in the HME cells with an IC_{50} of about 0.1 μM, a concentration that should be possible to achieve from the normal dietary content of this flavonoid.

Because we have determined that EST catalyzes the bioactivation of the cooked-food mutagen and breast carcinogen N-OH-PhIP, promoting its subsequent binding to DNA, inhibiting EST with quercetin and other dietary flavonoids could serve as a protective mechanism in breast cancer initiation. As we have established the ability of flavonoids to inhibit EST in intact cells using HME cells as a model, we will work on determining if flavonoids can inhibit EST-mediated bioactivation of N-OH-PhIP using a DNA binding assay.

Overall we feel that the last year has been productive and has resulted not only in publications, but also more importantly in grant applications. The localization studies proposed in Task #1 have given us an opportunity to collaborate and receive input from pathologists and specialist in immunolocalization. Our findings in Task #2 have also led to a side project of trying to understand why Quercetin inhibition of EST in the intact cell is so much more potent compared to the recombinant enzyme. We believe that in the breast cell quercetin may be activated to a more potent form, inhibit synthesis of the cofactor PAPS, or perhaps most likely, inhibit some signaling pathway important for the regulation of EST expression.

Key Research Accomplishments:

- We localized EST to the epithelial cells lining ducts and alveoli of normal human breast tissue using immunohistochemistry. Previously, EST had only been isolated from cultured human breast cells and detected by immunoblot analysis.

- We localized EST to the nuclei of normal human mammary epithelial cells using immunostaining with confocal microscopy. This is a key finding in that nuclear EST may represent a novel EST isoform or EST may have a different subcellular localization than currently believed.

- We determined that EST expression increases with increasing days following cell seeding.

- We have established that the flavonoids quercetin and resveratrol potently inhibit isolated recombinant human EST in a competitive fashion.

- We have established that quercetin and resveratrol also potently inhibit EST activity in the intact human mammary epithelial cells and that the inhibition by quercetin is surprisingly more potent in the intact cell compared to the recombinant enzyme.

Reportable Outcomes:

1. Lewis, AJ, Walle, UK, King, RS, Kadlubar, FF, Falany, CN, and Walle, T. (1998) Bioactivation of the cooked food mutagen N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine by estrogen sulfotransferase in cultured human mammary epithelial cells. *Carcinogenesis*, **19**, 2049-2053.

2. Lewis-Nolan, A, Otake, Y, Walle, UK, and Walle, T. (2000). Inhibition of estrogen sulfotransferase in normal human mammary epithelial cells. *Proceedings of the American Association for Cancer Research*. **41**, 836.

3. Otake, Y, Lewis-Nolan A., Walle, UK, and Walle, T. (2000). Quercetin and resveratrol potently reduce estrogen sulfotransferase activity in normal human mammary epithelial cells. *The Journal of Steroid Biochemistry and Molecular Biology* **73**, 265-270.

4. *DOD concept grant application*: Estrogen sulfotransferase, a marker for breast cancer progression, submitted 4/12/00

5. *DOD idea grant application*, Estrogen sulfotransferase, a key enzyme in breast cancer development, submitted 6/7/00

Carcinogenesis vol.19 no.11 pp.2049–2053, 1998

SHORT COMMUNICATION

Bioactivation of the cooked food mutagen *N*-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by estrogen sulfotransferase in cultured human mammary epithelial cells

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Cooked food mutagens from fried meat and fish have recently been suggested to contribute to the etiology of breast cancer. Thus, the most prevalent of these compounds, i.e. 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, or rather its more mutagenic *N*-hydroxylated metabolite (N-OH-PhIP), forms DNA adducts in mammary cells, including human mammary epithelial (HME) cells. The objective of this study was to determine the involvement of estrogen sulfotransferase (EST), the only sulfotransferase identified in HME cells, in the further bioactivation of N-OH-PhIP. These studies were done *in vitro* using human recombinant EST and in intact HME cells. Human recombinant EST increased the covalent binding of [^{3}H]N-OH-PhIP to calf thymus DNA ~3.5-fold in the presence of the sulfotransferase co-substrate 3′-phosphoadenosine-5′-phosphosulfate at each N-OH-PhIP concentration (1, 10 and 100 μM) ($n = 6$, $P < 0.001$). In contrast, EST did not catalyze the DNA binding of two other cooked food mutagens, *N*-hydroxy-2-amino-3-methylimidazo[4,5-*f*]quinoline and *N*-hydroxy-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, which are mainly hepatocarcinogens. Cultured HME cells displayed high EST activity, which could be completely inhibited by 1 μM estrone. When the cells were incubated with [^{3}H]N-OH-PhIP, binding to native DNA occurred at 60–240 pmol/mg DNA. This binding was inhibited to 55% of control by 1 μM estrone ($P < 0.01$, $n = 8$), suggesting that EST plays a significant role in carcinogen bioactivation in human breast tissue.

Breast cancer is the second leading cause of cancer-related death in American women (1), yet its etiology remains unknown. Factors such as family history and lifetime exposure to endogenous estrogens account for ~30% of breast cancer cases (2). Studies suggest that exogenous genotoxic carcinogens present in our diet and the environment may contribute to human breast cancer (3). It has been suggested that the highest cancer risks may result from ingestion of fried beef and fish products (4–6). 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), the most prevalent of the heterocyclic amines formed during the cooking process of various meats (4,7), is a mutagen in *Salmonella typhimurium* (8) and Chinese hamster ovarian cells (9,10). *In vivo* PhIP has been shown to induce both colon and mammary tumors in rodents (11,12). Although most studies have focused on the role of PhIP as a colon carcinogen, it also should be important to determine its potential involvement in human mammary tumor initiation.

Several recent studies suggest that normal human mammary epithelial (HME) cells may have the capacity to activate several carcinogens, one being PhIP, to species capable of binding DNA (13–15). In order for PhIP to bind to DNA, it must first be *N*-hydroxylated by cytochrome P450 1A2 (16,17). Furthermore, the *N*-hydroxylated metabolite is then converted to its ultimate carcinogen via phase II metabolizing enzymes, most prominently the acetyltransferases or sulfotransferases (15,18–26).

The finding that HME cells express only one of multiple isoforms of the sulfotransferase family, i.e. estrogen sulfotransferase (EST) (27), was of particular interest. This isoform has substrate specificities similar to that of the phenol form of sulfotransferase (P-PST) (28), previously shown to activate *N*-hydroxy PhIP (N-OH-PhIP) (21,25,26). The objective of this study was to determine the ability of EST to sulfate N-OH-PhIP and two other cooked food mutagens, *N*-hydroxy-2-amino-3-methylimidazo[4,5-*f*]quinoline (N-OH-IQ) and *N*-hydroxy-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (N-OH-MeIQx), to species capable of binding to DNA. These studies were carried out both *in vitro*, using human recombinant EST, and in cultured HME cells.

[^{3}H]N-OH-PhIP (101 mCi/mmol) (20), [^{3}H]N-OH-IQ (99 mCi/mmol) and [^{3}H]N-OH-MeIQx (130 mCi/mmol) were prepared as previously described (19,29). Ultrapure 3′-phosphoadenosine-5′-phosphosulfate (PAPS) was purchased from S.S.Singer (University of Dayton, Dayton, OH). [^{3}H]PhIP (2.15 Ci/mmol) was obtained from Chemsyn Science Laboratories (Lenexa, KS) through the National Cancer Institute Chemical Carcinogen Reference Standard Repository. All other chemicals were purchased from Sigma (St Louis, MO).

Recombinant human EST was isolated and purified from EST/pKK233-2 XL1-Blue cultures after induction with 1 mM isopropyl-β-D-thiogalactopyranoside for 5 h at 37°C, as previously described (28) with several modifications (30). The isolated enzyme preparation was free of other sulfotransferases but did contain bacterial proteins. Purification of EST to homogeneity was unsuccessful due to instability of the enzyme. The actual concentration of EST is therefore not known. It sulfated its natural substrate β-estradiol with a K_m value of 25 nM, which is similar to a previous report (28).

The EST-catalyzed sulfation of the *N*-hydroxylated amines was measured as the PAPS-dependent covalent binding to calf thymus DNA of the labile sulfuric acid ester formed, using radiolabeled substrates, as previously described for P-PST

Abbreviations: EST, estrogen sulfotransferase; HBSS, HEPES-buffered balanced salt solution; HME, human mammary epithelial; N-OH-IQ, *N*-hydroxy-2-amino-3-methylimidazo-[4,5-*f*]quinoline; N-OH-MeIQx, *N*-hydroxy-2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline; N-OH-PhIP, *N*-hydroxy-PhIP; NAT-1, N-acetyltransferase 1; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; P-PST, phenol form of phenolsulfotransferase.

(26). Conditions for the reaction were optimized using 20 nM [^{3}H]β-estradiol (28). Saturating concentrations of co-substrate were reached at 20 μM PAPS. Sulfation was linear with time up to 60 min and with enzyme up to 6 μl EST preparation.

The typical reaction mixture consisted of calf thymus DNA (2 mg/ml), recombinant human EST and 20 μM PAPS in a final incubation volume of 100 μl 33 mM Tris buffer (pH 7.5). The reactions were initiated with [^{3}H]N-OH-PhIP, [^{3}H]N-OH-IQ or [^{3}H]N-OH-MeIQx (1, 10 and 100 μM) and were incubated at 37°C for 30 min under argon saturation. The radiolabeled drugs were added in dimethyl sulfoxide:ethanol (4:1). Control incubates did not contain the co-substrate PAPS. After incubation, the samples were extracted twice with *n*-butanol saturated with distilled water and once with phenol: chloroform:isoamyl alcohol (25:24:1, pH 8) (Amersco, Solon, OH) with 1 g/l 8-hydroxyquinoline. DNA was then precipitated with 5 M sodium acetate and 100% ethanol. The DNA pellet was washed three times with ethanol, resuspended in Tris buffer and analyzed by liquid scintillation spectrometry. Washes were counted to ensure removal of non-specific binding. DNA recovery was determined by measuring the UV absorbance at 260 nm. On average, 90% of the DNA was recovered.

Primary HME cells, at passage 7, were obtained from Clonetics (San Diego, CA). These cells were derived from a 22-year-old healthy woman who had undergone reduction mammoplasty. Cell cultures were maintained as recommended by Clonetics. Serum-free mammary epithelial growth medium was supplemented with the following (final concentrations): bovine pituitary extract (52 μg/ml), human recombinant epidermal growth factor (10 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), gentamicin (50 μg/ml) and amphotericin-B (50 ng/ml). Experiments were conducted with cells at passage 9–11.

HME cells grown to confluent monolayers on 100 mm plates were washed twice with a HEPES-buffered balanced salt solution (HBSS) of the following composition: 10 mM glucose, 20 mM HEPES, 1.2 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and NaOH to a pH of 7.4. The cells were then incubated for 4 h at 37°C with 10 μM [^{3}H]N-OH-PhIP in 5 ml HBSS in the presence or absence of 1 μM estrone. The incubation buffer was removed and cells were lifted by incubating them for 5 min at 37°C in 10 mM Tris buffer (pH 8) containing 1 mM EDTA and 0.14 M NaCl. Cells were collected by centrifugation and incubated overnight at 37°C in 10 mM Tris buffer (pH 8) containing 0.1 M EDTA, 100 μg/ml proteinase K and 0.5% SDS. The mixture was then extracted with 3 vols phenol:chloroform:isoamyl alcohol. DNA and RNA present in the aqueous phase were precipitated with 1 vol cold 100% ethanol and 5 M sodium acetate. The pellet was resuspended in 10 mM Tris buffer (pH 8) containing 0.1 M EDTA and 20 μg/ml pancreatic RNase and incubated for 1 h at 37°C. After extraction with 3 vols phenol:chloroform:isoamyl alcohol, DNA was again precipitated with 100% ethanol, collected by centrifugation and dissolved in water. DNA recovery was determined by UV absorbance at 260 nm. The amount of [^{3}H]N-OH-PhIP bound to DNA was quantitated by liquid scintillation spectrometry.

Results are expressed as mean values ± SE. The statistical significance of differences was analyzed by Student's *t*-test for unpaired data. Because of the non-normal distribution of the whole cell data, the Mann–Whitney test was used.

The EST-catalyzed binding of [^{3}H]N-OH-PhIP to calf thymus DNA is shown in Figure 1. Figure 1A shows the binding of increasing concentrations of [^{3}H]N-OH-PhIP to DNA in incubates containing 6 μl EST. The control binding, i.e. without the co-substrate PAPS, represents non-enzymatic binding of the *N*-hydroxylamine directly to DNA. With catalytically active EST, i.e. in the presence of the co-substrate PAPS, binding of N-OH-PhIP to calf thymus DNA increased ~3.5-fold at each N-OH-PhIP concentration (1, 10 and 100 μM) ($n = 6$ at each concentration; $P < 0.001$). Figure 1B shows the binding of one concentration of [^{3}H]N-OH-PhIP (100 μM) to DNA at various EST concentrations. The binding increased with increasing enzyme concentrations. Whereas the control binding (without PAPS) stayed the same and was subtracted for each enzyme concentration, the PAPS-dependent binding was linear with amount of enzyme ($y = 6.13x + 3.12$, $r = 1.000$).

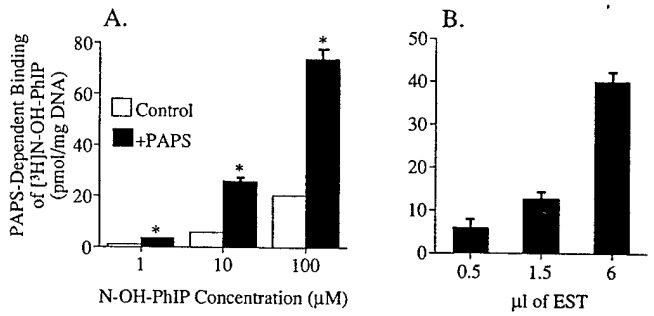

Fig. 1. (**A**) Binding of increasing concentrations of [^{3}H]N-OH-PhIP to calf thymus DNA in incubates with 6 μl recombinant human EST. The open bars (Control) are in the absence of the co-substrate PAPS and the closed bars (+PAPS) in the the presence of 20 μM PAPS. (**B**) Binding of 100 μM [^{3}H]N-OH-PhIP to calf thymus DNA in incubates with increasing amounts of recombinant human EST. Each bar represents binding in the presence of 20 μM PAPS with the control binding, i.e. without PAPS, subtracted. Data are expressed as means ± SE ($n = 6$). *Significantly higher than for the control ($P < 0.001$).

2-Naphthylamine is a substrate for sulfoconjugation, resulting in formation of a sulfamate (31). PhIP itself, a primary heterocyclic amine with aromatic properties, might thus also be sulfated without prior oxidation. However, EST possessed no ability to bioactivate [^{3}H]PhIP to a species capable of binding DNA. There was thus no increased binding to DNA in the presence of PAPS at either 1, 10 or 100 μM [^{3}H]PhIP ($n = 6$ at each concentration). Not unexpectedly, the background binding of [^{3}H]PhIP was 20-fold lower compared with [^{3}H]N-OH-PhIP.

In Figure 2 is shown the binding of [^{3}H]N-OH-IQ and [^{3}H]N-OH-MeIQx to calf thymus DNA, using incubation conditions identical to those in Figure 1A. In sharp contrast to N-OH-PhIP, catalytically active EST did not significantly enhance the binding of N-OH-IQ to DNA compared with control incubates. A minimal (15–20%) but statistically significant increase in DNA binding of N-OH-MeIQx in the presence of PAPS and EST was observed at 10 and 100 μM substrate concentrations, but not at 1 μM.

As can be seen in Figure 2, N-OH-MeIQx had similar non-enzymatic background binding as N-OH-PhIP, whereas that for N-OH-IQ was several times higher. This is consistent with a previous study (19). Also, for all three *N*-hydroxylated heterocyclic amines the DNA binding in the absence of EST was identical to that in the absence of PAPS.

These findings suggest that EST plays a role in the conversion of N-OH-PhIP, a potent mammary carcinogen, to a more

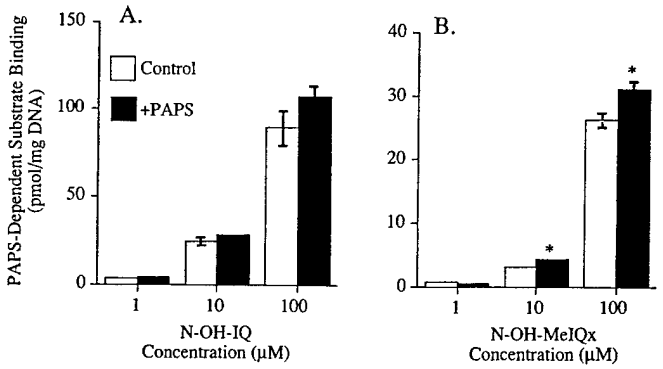

Fig. 2. Binding of increasing concentrations of [^{3}H]N-OH-IQ (**A**) and [^{3}H]N-OH-MeIQx (**B**) to calf thymus DNA in incubates with 6 μl recombinant human EST. The open bars (Control) are in the absence of the co-substrate PAPS and the closed bars (+PAPS) in the presence of 20 μM PAPS. Data are expressed as means ± SE (n = 6). *Significantly higher than for the control ($P < 0.001$),

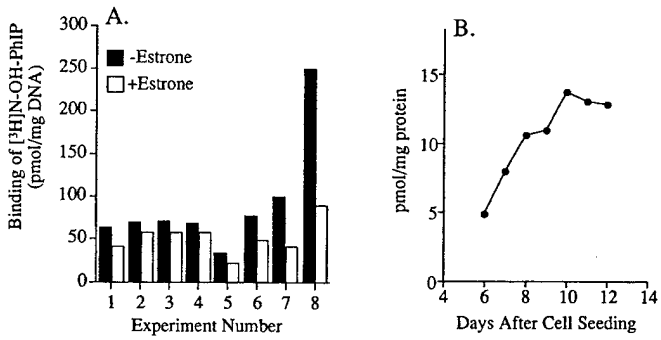

Figure 3. (**A**) Binding of 10 μM [^{3}H]N-OH-PhIP to native DNA of normal HME cells in eight individual experiments. The closed bars are in the absence of inhibitor and the open bars are in the presence of 1 μM estrone. (**B**) EST activity in HME cells at different times after cell seeding in a separate set of experiments. EST activity was measured over 3 h with β-estradiol as substrate.

reactive species. It is of interest to notice that N-OH-MeIQx and N-OH-IQ, mainly hepatocarcinogens (32,33), are not further bioactivated by EST. However, these carcinogens may be better substrates for other bioactivating enzymes, such as the acetyltransferases (19,22). The high level of acetyltransferase activity in the liver may play a role in the tissue-specific carcinogenicity of IQ, the most potent rat and primate hepatocarcinogen described (22,33). Conversely, the higher affinity of N-OH-PhIP for EST expressed in normal breast cells may contribute to the tissue-specific carcinogenicity of PhIP in the mammary gland.

In order to assess the importance of EST-catalyzed N-OH-PhIP bioactivation under more physiological conditions, we used intact cultured HME cells as the model system. EST is the only sulfotransferase expressed in these cells, as determined by western blotting (27). The EST activity in HME cells, determined with 20 nM [^{3}H]β-estradiol as substrate at 6 days after cell seeding, was 5.1 ± 1.0 pmol/mg cellular protein for a 3 h incubation, which is 2–3 times that reported previously at 1 day after cell seeding (27). To be able to use this model system in our studies, a specific EST inhibitor was obligatory. Estrone, by virtue of being another high affinity substrate for EST, provided this tool, producing virtually complete inhibition of β-estradiol sulfation by HME cells at a concentration as low as 1 μM. Using an established human red blood cell assay (34), estrone did not inhibit NAT-1, the other potential bioactivating enzyme (13), even at a concentration of 1000 μM.

To assess EST-dependent binding of [^{3}H]N-OH-PhIP to native DNA of the intact cell, HME cells were treated with 10 μM [^{3}H]N-OH-PhIP, the intermediate concentration in Figure 1A, for 4 h in the presence and absence of 1 μM estrone. Cellular DNA was then isolated and purified as described in Materials and methods and analyzed for incorporation of radiolabeled N-OH-PhIP. Figure 3A shows the EST-catalyzed binding of [^{3}H]N-OH-PhIP to native DNA of HME cells in eight individual experiments. In all experiments estrone reduced binding of N-OH-PhIP to DNA. Mean binding in the presence of the inhibitor was 55% of control ($P < 0.01$, n = 8). The variability between individual experiments appears to reflect a difference in EST expression. Thus, in experiments 5–8, performed in the same batch of cells at 6, 8, 9 and 10 days post-cell seeding, there was an EST-mediated binding of 11.8, 29.0, 58.8 and 160.0 pmol [^{3}H]N-OH-PhIP/mg DNA, respectively, i.e. markedly increased binding with increased maturity of the HME cells. A separate set of experiments examined EST expression in the HME cells at various times post-cell seeding. As shown in Figure 3B, there was an almost linear increase in EST activity over days 6–10, correlating well with the increased estrone-inhibitable DNA binding of [^{3}H]N-OH-PhIP. It is obvious that estrone did not completely inhibit the binding of [^{3}H]N-OH-PhIP to native HME cell DNA. This non-EST-mediated binding may be attributed to direct binding of [^{3}H]N-OH-PhIP, as seen in Figure 1A, or its potential oxidation products. It should be noted that the incubations with recombinant EST were carried out under argon to protect the N-OH-PhIP from oxidative degradation, which obviously could not be employed with intact cells. Another contributor to this binding could be bioactivation of N-OH-PhIP by another enzyme, such as acetyltransferase. Sadrieh *et al.* (15), using the ^{32}P-post-labeling technique, have indeed demonstrated with human mammary gland cytosol, although not with intact HME cells, that *N*-acetyltransferase 1 (NAT-1) *O*-acetylates N-OH-PhIP, increasing its reactivity towards DNA. It therefore appears feasible to postulate that both EST and NAT-1 present in normal human breast cells may further bioactivate N-OH-PhIP, promoting DNA adduct formation that, if unrepaired (13), may lead to tumor initiation.

It is not known if these cooked food heterocyclic amines are hydroxylated in the liver and then transported via the blood to their target sites for further enzymatic activation or if both oxidation and phase II esterification occur at the site of tumorigenicity. Whereas PhIP is the promutagen ingested with cooked foods (8), N-OH-PhIP is derived mainly from cytochrome P450 1A2 oxidation in the body. Although most of this oxidation probably takes place in the liver (16,19), it may also occur in breast cells. It has been suggested that DNA adducts are formed in HME cells when the cells are exposed to the promutagen PhIP (14). Although cytochrome P450 1A2 is not expressed in breast cells, a variety of other cytochrome P450 isoforms have recently been detected (35). In fact, a recent report shows that PhIP is a substrate for cytochrome P450 1B1, a newly described P450 present in normal human breast cells (36).

However, even if the generation of N-OH-PhIP from PhIP is not efficient in breast cells, N-OH-PhIP could be transported from the liver. Further bioactivation could then occur in the breast cells, as previously suggested (13). Although this might include *O*-acetylation (13,15), our present study suggests that this bioactivation also includes sulfation mediated by EST.

It is known that PhIP induces mutations in a tumor suppressor gene, *APC*, associated with colorectal cancer development (7,37). While mutations in various genes, including *ras* and *p53*, have been identified in PhIP-induced mammary tumors (38,39), the principal genes containing mutations are still an area of investigation.

EST is the only human sulfotransferase that has been shown to be hormonally regulated. Using an endometrial adenocarcinoma cell line, Falany and Falany found that progesterone increases EST expression as much as 7-fold (40). During the luteal phase of the menstrual cycle there is a surge in progesterone levels and one may speculate that during this time women may be more susceptible to carcinogen bioactivation through EST.

In conclusion, human EST has been shown to be capable of sulfoconjugating N-OH-PhIP. This reaction was shown to increase the binding of N-OH-PhIP to calf thymus DNA by >3-fold. Extending these studies to the more physiological intact cultured HME cells clearly demonstrated EST-catalyzed binding of N-OH-PhIP to native cellular DNA. As EST is the only sulfotransferase isoform expressed in normal human breast cells, we hypothesize that this reaction significantly contributes to the initiation of breast cancer in humans.

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Quercetin and resveratrol potently reduce estrogen sulfotransferase activity in normal human mammary epithelial cells

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Abstract

Estrogen sulfotransferase (EST) is the sole sulfotransferase expressed in normal human breast epithelial cells and has an important function in determining free estrogen hormone levels in these cells. In the present study we examined the inhibitory effect of the dietary polyphenols quercetin and resveratrol on EST activity, i.e. 17β-estradiol (E2) sulfation. Both the compounds potently inhibited recombinant human EST in a competitive fashion with K_i values of about 1 μM. In fact, both polyphenols could serve as substrates for EST. In order to extend the studies to more physiologically relevant conditions, we examined whether inhibition of EST also occurred in the intact cultured human mammary epithelial (HME) cells. The mean baseline EST activity (E2 sulfate formation) in the HME cells was 4.4 pmol/h per mg protein. The IC_{50} for resveratrol was very similar to that for recombinant EST, i.e. about 1 μM. Surprisingly, quercetin was 10 times more potent in the HME cells with an IC_{50} of about 0.1 μM, a concentration that should be possible to achieve from the normal dietary content of this flavonoid.

Keywords: hEST; Estrogen sulfotransferase; Human mammary epithelial cells; Quercetin; Resveratrol; Polyphenols

1. Introduction

Estrogen hormones are important for the growth and development of normal human breast tissue. The presence of estrogen, however, is also a requirement for the growth and development of most breast cancers. Although the major proportion of active estrogen in premenopausal women is produced by the ovaries, and thus requires transport to the breast tissue, an increasing proportion is synthesized in peripheral tissues, including the breast [1,2], in postmenopausal women. Biological processes that affect the intracellular availability of estrogen hormones to their receptors will, therefore, influence the progression of breast cancer. This has led to the development of multiple strategies to decrease the cellular exposure and response to estrogen hormone. Two therapeutically accepted approaches to accomplish this are inhibition of estrogen action by antiestrogens, which interact with estrogen receptors [3], and inhibition of estrogen production by inhibitors of aromatase, the enzyme responsible for estrogen synthesis [4].

The intracellular levels of estrogen can also be affected by conjugating enzymes, e.g. UDP-glucuronosyl transferase [5], and in particular estrogen sulfotransferase (EST), an enzyme highly specific for 17β-estradiol (E2) and estrone [6,7]. Interestingly, EST has been demonstrated to be highly expressed in the normal human mammary epithelial cells, but with very low or no expression in breast cancer cells [6,7]. A high EST expression, which can be stimulated by progesterone [8], may result in diminished estrogen hormone levels and a protective effect [6–8]. The resulting estrogen sulfates can, however, be hydrolyzed by estrogen sulfatase [2,9]. A recent study indicated that dietary flavonoids, including quercetin, may inhibit estrogen sulfatase, suggesting a protective effect of these dietary polyphenols [10].

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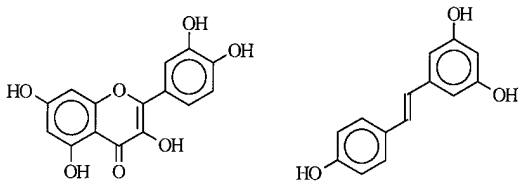

Fig. 1. Chemical structures of the dietary polyphenols quercetin and resveratrol.

Previous studies have demonstrated that flavonoids can be potent inhibitors of a human sulfotransferase, P-PST [11,12], which can sulfonate high concentrations of estrogen hormones [13]. In the present study we demonstrate very potent inhibition of EST by the polyphenols quercetin and resveratrol (Fig. 1), using both recombinant EST, as well as cultured normal human mammary epithelial (HME) cells.

2. Materials and methods

2.1. Materials

E2, quercetin, quercitrin, and resveratrol were purchased from Sigma (St. Louis, MO). [^{3}H]Estradiol ([^{3}H]E2) (48 Ci/mmol) and [^{35}S]-labeled 3′-phosphoadenosine-5′-phosphosulfate (PAPS) (1.0–1.5 Ci/mmol) were purchased from DuPont-New England Nuclear (Wilmington, DE). Ultrapure PAPS was purchased from Dr S.S. Singer (University of Dayton, Dayton, OH). Quercetin 4′-monoglucoside was purified from red onions as previously described [14].

2.2. Isolation of recombinant EST

EST cDNA subcloned into the pKK233-2 bacterial vector and expressed in *Escherichia coli* XL1-Blue strain was donated by Dr C.N. Falany (University of Alabama at Birmingham). Recombinant human EST was isolated and purified from EST/pKK233-2 XL1-Blue cultures after induction with 1 mM isopropyl-β-D-thiogalactopyranoside for 5 h at 37°C, as previously described [15] with several modifications [16].

2.3. In vitro incubations

E2 sulfation was assayed as previously described [15] using a chloroform extraction procedure (pH 8) [17]. The reaction mixtures containing 0.01 μl human recombinant EST, 20 nM [^{3}H]E2 and 25 μM PAPS in Tris–HCl buffer, pH 7.4, with 8 mM dithiothreitol and 0.0625% BSA in a final volume of 200 μl, were incubated at 37°C for 30 min. Reactions were performed in the presence and absence of the inhibitors quercetin, quercetin 4′-monoglucoside, quercitrin, and resveratrol (0.1–100 μM). The reactions were terminated by adding 250 μl 0.25 M Tris–HCl buffer, pH 8.7, and 3 ml chloroform. After mixing and centrifugation, aliquots of the aqueous phase were subjected to liquid scintillation counting. To determine the mode of EST inhibition by quercetin and resveratrol, a range of [^{3}H]E2 concentrations (5–50 nM), as well as inhibitor concentrations (0.5–2 μM) were used.

2.4. Culturing of human mammary epithelial (HME) cells

Primary human mammary epithelial (HME) cells, at passage 7, were obtained from Clonetics (San Diego, CA). These cells were derived from a 22-year-old healthy woman who had undergone reduction mammoplasty. Cell cultures were maintained as recommended by Clonetics [18]. Serum-free mammary epithelial growth medium was supplemented with the following (final concentrations) bovine pituitary extract (52 μg/ml), human recombinant epidermal growth factor (10 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), gentamicin (50 μg/ml), and amphotericin B (50 ng/ml). Experiments were conducted with cells at passage 9–11.

2.5. In situ HME cell incubations

For studies of E2 sulfation in intact HME cells, cells were seeded in 6-well plates and allowed to grow to confluency (8–10 days) [18]. The cells were then incubated for 1 h at 37°C with 20 nM [^{3}H]E2 in 1 ml of a HEPES-buffered balanced salt solution (HBSS) of the following composition: 10 mM glucose, 20 mM HEPES, 1.2 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and NaOH to a pH of 7.4. Incubations were performed in the presence and absence of the inhibitors quercetin and resveratrol (0–10 μM). 100 μl aliquots of the HBSS incubation buffer were sampled at various times and E2 sulfate formation was assayed as previously described [15] using the alkaline-chloroform extraction procedure [17].

2.6. Sulfation of quercetin and resveratrol

EST-catalyzed sulfation of quercetin and resveratrol was determined using the previously described ion-pair extraction method [19]. The typical reaction mixture contained 0.1–10 μM of the polyphenol substrate, 1 μM [^{35}S]PAPS and 0.1 μl of recombinant EST in 33 mM Tris–HCl buffer, pH 7.4, with 8 mM dithiothreitol and 0.0625% BSA in a total volume of 100 μl. The samples were incubated for 30 min at 37°C and the reactions were terminated by the addition of 10 μl 2.5% acetic acid, 20 μl of 0.1 M tetrabutylammonium hydrogen sulfate and 500 μl ethyl acetate. After mixing and

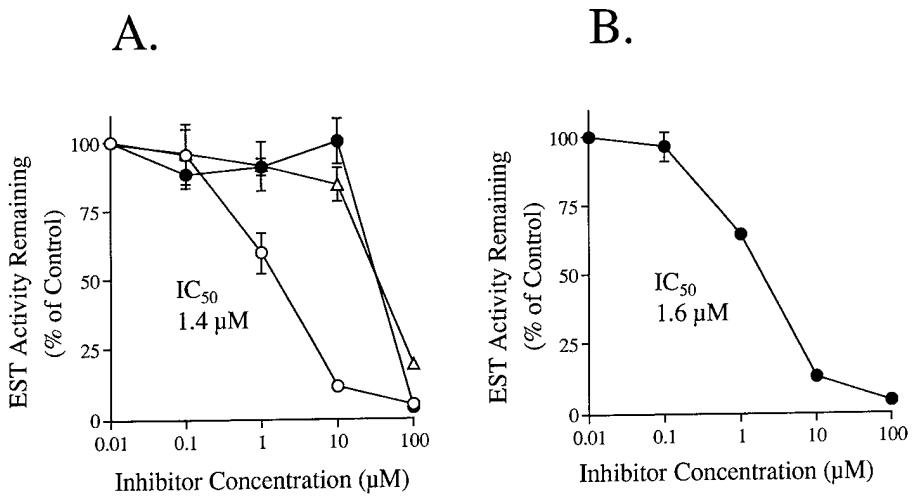

Fig. 2. Inhibition of E2 sulfation by (A) quercetin (○), quercetin 4′-monoglucoside (●) and quercitrin (△) and (B) resveratrol, using recombinant human EST. Experiments are mean ± S.E.M. ($N = 3$). For some points the error bars are smaller than the symbols.

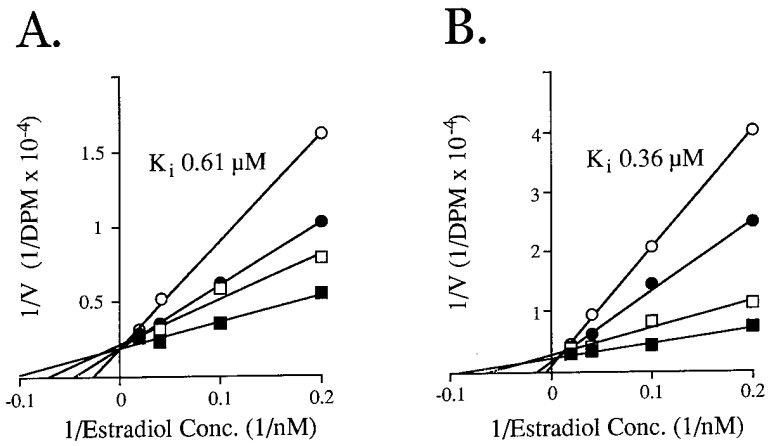

Fig. 3. Inhibition of E2 sulfation by (A) quercetin and (B) resveratrol, using recombinant EST. In these double reciprocal plots of velocity versus substrate concentration the inhibitor concentrations used were 0 (■), 0.5 (□), 1 (●) and 2 (○) μM. The E2 concentrations used were 5–50 nM. The data represent mean values of two experiments.

centrifugation, 400 μl of the ethyl acetate extract was subjected to liquid scintillation counting.

2.7. Data analysis

The K_m values were derived with UltraFit (Biosoft, Cambridge, UK) from V versus S plots, using the Michaelis-Menten equation [20]. The IC_{50} values for the concentration-activity curves from individual experiments were derived with UltraFit, using an equation for double exponential decay with offset. The mode of quercetin and resveratrol inhibition of E2 sulfation by recombinant EST and its K_i values were derived graphically from Lineweaver–Burk plots (1/V vs. 1/S) and replots of the slope versus inhibitor concentration [21].

3. Results

The recombinant human EST was isolated as described [15] and characterized catalytically with its main natural substrate E2. The formation of E2 sulfate had an apparent K_m value of 21 nM. Saturating concentrations of the co-substrate PAPS were reached at 10 μM.

The sulfation of E2 by recombinant EST was inhibited potently by quercetin, yielding an IC_{50} value of 1.4 μM. The naturally occurring glycosides of quercetin, i.e. quercetin 4′-glucoside and quercitrin (quercetin 3-rhamnoside) were considerably less potent, with estimated IC_{50} values of about 30 μM, Fig. 2A. Resveratrol had a potency very similar to that of quercetin with an IC_{50} value of 1.6 μM, Fig. 2B.

To determine the mode of inhibition, varying concentrations of quercetin and resveratrol were used (0.5–2 μM) together with varying concentrations of the substrate E2 (5–50 nM). As shown in Fig. 3A and B, both quercetin and resveratrol appeared to be competitive inhibitors of E2 sulfation. The K_i values, although somewhat lower than the IC_{50} values in Fig. 2, were very similar for quercetin and resveratrol, 0.58 and 0.36 μM, respectively.

The finding that the inhibition was competitive suggested that both quercetin and resveratrol are substrates for the human EST. This was tested using an ion-pair extraction method [19], particularly suitable for labile sulfate conjugates. The data shown in Fig. 4A and B clearly demonstrated that both quercetin and resveratrol are sulfated, with apparent K_m values of 0.32 and 0.53 μM, respectively, i.e. very similar to their K_i values for inhibition of E2.

To determine the potential physiological significance of EST inhibition by quercetin and resveratrol, experiments were carried out in cultured intact human mammary epithelial (HME) cells. These cells have previously been shown to express high EST activity [6,18]. The mean baseline EST activity of the HME cells in these experiments was 4.4 pmol of E2 sulfate formed per hr and mg protein, using 20 nM E2. Surprisingly, the quercetin inhibition of E2 sulfation in the intact HME cells was more potent than by recombinant EST, yielding an IC_{50} value as low as 0.13 μM, Fig. 5A. Complete inhibition occurred at 1 μM quercetin. For resveratrol, the inhibition was similar to that observed with recombinant EST, resulting in an IC_{50} value of 1.3 μM, Fig. 5B.

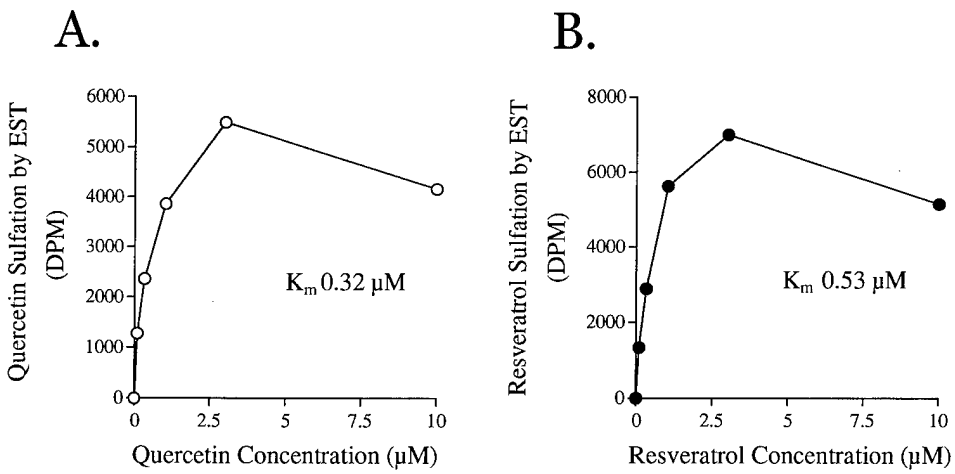

Fig. 4. EST-catalyzed sulfation of (A) quercetin and (B) resveratrol. The data represent mean values of two determinations.

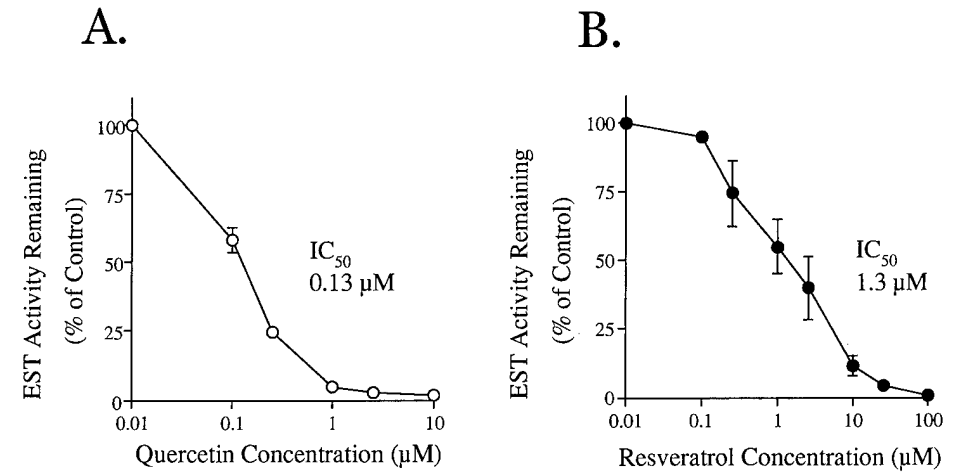

Fig. 5. Inhibition of E2 sulfation by (A) quercetin and (B) resveratrol in the cultured intact HME cells. The cells were incubated for 3 h with [^{3}H]E2 in the absence and presence of varying concentrations of inhibitor. [^{3}H]E2 sulfate was then measured in the medium. The data shown represent mean ± S.E.M. of three experiments. For some points the error bars are smaller than the symbols.

4. Discussion

Quercetin and resveratrol demonstrated very similar inhibition of the catalytic activity of the recombinant human EST with an IC_{50} value of about 1 μM, whereas two of the naturally occurring glycosides of quercetin were about 30 times less potent. Interestingly, the inhibition by both quercetin and resveratrol appeared to be simply a competitive type of interaction. Therefore, as expected, both of these dietary components were substrates for EST, with K_m values similar to their K_i values for inhibition of E2 sulfation. This finding is interesting in that P-PST, the most ubiquitous of the human STs, responsible for the sulfation of most foreign phenolic compounds [22] and highly related to EST [15], does not appear to use quercetin as a substrate [12].

Quercetin has previously been shown to also inhibit the catalytic activity of P-PST with an IC_{50} value as low as 0.1 μM [11,12]. This inhibition was noncompetitive in nature. In the intact human hepatoma cell line Hep G2, which has P-PST expression similar to the human liver [23], the potency of quercetin inhibition of P-PST decreased about 25-fold, yielding an IC_{50} value of 2.5 μM [12]. This was speculated to be due to a combination of factors, including poor plasma membrane penetrability as well as extensive metabolism of quercetin. This was thought to be consistent with the generally held view that flavonoids have a low cellular availability.

The very potent inhibitory effect of quercetin on the sulfation of estradiol by EST in the intact HME cells is therefore most surprising. Thus, quercetin is 25 times more potent inhibiting the EST activity in these cells than inhibiting the P-PST activity in Hep G2 cells, even though quercetin is 10 times less potent inhibiting recombinant EST than P-PST. The mechanism for this potent inhibition is unclear. It could involve (1) a mechanism concentrating quercetin inside the breast cell, (2) bioactivation to a more potent form, e.g. by *O*-methylation [24,25], (3) inhibition of synthesis of the cofactor PAPS, or perhaps most likely, (4) inhibition of some signaling pathway important for the regulation of EST expression. The IC_{50} of 0.1 μM corresponds to a quercetin concentration of about 30 ng/ml, which is five to ten times lower than concentrations in plasma reported in humans after consuming common foodstuffs rich in quercetin, such as onions and apples [26]. Even when taking into account the high plasma binding of quercetin [27], this potent effect on EST function in the breast cell may be relevant in humans.

Even though inhibition of EST by quercetin, resulting in elevated estrogen hormone levels in the normal breast cell, may be a potentially harmful effect, other considerations may be of importance. In a recent study, it was demonstrated, also in the HME cells, that EST could catalyze the bioactivation of the cooked-food mutagen and procarcinogen *N*-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-OH-PhIP) and its subsequent binding to genomic DNA [18]. This potential breast cancer initiating reaction was highly correlated to EST expression. Thus, inhibiting EST with quercetin and other dietary flavonoids could serve as a protective mechanism in breast cancer initiation. Although inhibition of EST may increase circulating levels of estradiol, quercetin has been shown to compete with a similar affinity as E2 for type II estrogen binding sites [28]. The interaction of quercetin at these sites serves to inhibit the estradiol induction of cellular proliferation [28–31]. Therefore, as quercetin may increase mean estradiol concentration by inhibiting EST, this potentially harmful effect may be countered by the opposing, growth inhibitory, action of quercetin at the type II estrogen binding sites.

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UGT1A6. Microsomes expressing UGT1A9 formed N^2-gluc and N3-gluc at levels similar to UGT1A4, however, N3-gluc was formed at a rate 3 times greater than N^2-gluc. ESI-MS analysis of all 3 metabolites produced molecular ions at *m/z* 417 $[M+H]^+$ and, upon MS/MS, N^2-gluc and the uncharacterized glucuronide fragmented to *m/z* 241 $[M+H]^+$ and N3-gluc fragmented to *m/z* 225 $[M+H]^+$. Upon treatment with β-glucuronidase only the N3-gluc was susceptible to enzymatic cleavage. The uncharacterized glucuronide has properties very similar to the N^2gluc. Studies are ongoing to fully characterize all the glucuronides detected in these experiments. This work was performed under the auspices of the US DOE by LLNL under contract #W-7405-ENG-48 and supported by NCI grant CA55861.

#5305 THE IMPACT OF A HIGH-FAT DIET ON THE CARCINOGENICITY AND LEVELS OF 8-HYDROXYDEOXYGUANOSINE (8-OHDG) IN THE MAMMARY GLANDS OF RATS TREATED WITH THE ENVIRONMENTAL POLLUTANT 6-NITROCHRYSENE (6-NC): A COMPARISON WITH 2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-*B*]PYRIDINE (PHIP). J Rosa, D Desai, T Boyiri, S Amin, K El-Bayoumy, X Gu, and J Schwartz, *American Health Fdn, Valhalla, NY, and Howard Univ, Potomac, MD*

The purpose of this study was two-fold: (1) to assess the impact of a high-fat (HF) diet on 6-NC-induced mammary cancer in female CD rats by oral administration and (2) to rank, under identical conditions, the carcinogenic potency of 6-NC and PhIP (an established mammary carcinogen) in the rat mammary gland. Thirty-day old female CD rats were fed a high-fat (23.5% corn oil) AIN-76A diet and gavaged once weekly for 8 weeks with various doses of 6-NC (50, 25, 12.5 μmol/rat/week in 0.5 ml trioctanoin). An additional group of rats were gavaged with 6-NC at the highest dose but were fed a low-fat (LF) diet (5% corn oil). PhIP was given at 50 μmol/rat/week for 8 weeks and animals were fed a high-fat diet. At termination (23 weeks after the last carcinogen dose) and on the basis of palpable mammary tumors, at the highest dose of 6-NC, the incidence and multiplicity were higher in rats fed a high-fat diet (93.3%, 3.57 tumors/rat) than those fed a low-fat diet (73.3%, 2.2 tumors/rat). Moreover, at equimolar doses, 6-NC appears to be a more potent mammary carcinogen (93.3%, 3.57 tumors/rat) than PhIP (72.4%, 2.10 tumors/rats). DNA samples were isolated from mammary tumors and non-involved tissue and analyzed by HPLC-EC detection. Levels of 8-OHdG were significantly higher in rats fed 6-NC + HF compared to those fed 6-NC + LF but similar to rats fed HF diet only. PCR-SSCP analysis showed a difference in Ha-*ras* mutational expressions between rats fed 6-NC + HF and 6-NC + LF. Although the role of a high-fat diet in human breast cancer remains controversial, the results of this study provide further support to the role of a high-fat diet as a promoter on experimental mammary carcinogenesis. Support: CA 35519.

#5306 DOSE AND TIME DEPENDENT EFFECTS OF THE FOOD MUTAGEN PHIP ON INTESTINAL TUMORIGENESIS IN *MIN*/+ MICE. Inger-Lise Steffensen, J. E Paulsen, A. Andreassen, R. Vikse, and J. Alexander, *National Institute of Public Health, Oslo, Norway*

The purpose of this work was to study in more detail how 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) affects intestinal tumorigenesis in the C57BL/6J- *Min*/+ (*M*ultiple *I*ntestinal *N*eoplasia) mice. *Min*/+ mice of both sexes were given a single s.c. injection of various doses of PhIP on days 3 – 6 or 11 – 13 after birth, or were exposed through their dams injected with PhIP 3 – 6 days before or after birth. The mice were terminated at eleven or seven weeks of age, and their small intestines and colons were fixed in 10% formalin and stained in methylene blue. The number, diameter and location of tumors were scored by transillumination in an inverse light microscope. After eleven weeks, there was a clear dose-response in the increased number and diameter of small intestinal tumors in mice injected on days 3 – 6 after birth with 10 or 50 mg/kg PhIP. The diameter, but not the number, of tumors was increased with 1 mg/kg PhIP. When terminated after 7 weeks, a similar dose-response was found, but the number and diameter of the tumors were lower. In the colon, there was a statistically non-significant tendency to increased number, but not diameter of tumors, with increasing doses. Exposure in utero 3 – 6 days before birth to 50 mg/kg PhIP increased the diameter, but not the number, of small intestinal tumors. However, 2.5 times higher number and even larger diameter of tumors were found in pups injected directly with 50 mg/kg PhIP on days 3 – 6 after birth compared with the same dose in utero. The number and diameter of the tumors were lower in pups injected with 50 mg/kg PhIP on days 11 – 13 versus on days 3 – 6 after birth. Apparently, there is a limited time-period of susceptibility, to PhIP in intestinal tumorigenesis. Exposure via milk from dams injected with 50 mg/kg PhIP on days 3 – 6 after birth increased the diameter, but not the number of small intestinal tumors.

#5307 THE METABOLISM OF 2-AMINO-3,8-DIMETHYLIMIDAZO[4,5-*F*]QUINOXALINE IN HUMAN HEPATOCYTES. S. Langouet, D. H Welti, N. Kerriguy, L. B Fay, J. Markovic, F. P Guengerich, A. Guillouzo, and Robert J Turesky, *INSERM U, Faculty de Pharmacie, Rennes, France, Nestle Res Ctr, Nestec Ltd, Lausanne, Switzerland, and Vanderbilt Univ Sch of Medicine, Nashville, TN*

The metabolism of the dietary mutagen 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) was studied in human hepatocytes. Six metabolites were characterized by ^{1}H NMR, LC-MS/MS and UV spectroscopy. The carcinogenic metabolite, HNOH-MeIQx, which is formed by P450 1A2, was present as the N^2-glucuronide conjugate. The Phase II conjugates MeIQx-N^2-sulfamate and MeIQx-N^2-glucuronide and the 7-oxo derivatives of MeIQx and N-demethyl-MeIQx were also identified. A novel metabolite was characterized as MeIQx-8-carboxylic acid and was a predominant metabolite formed in hepatocytes exposed to MeIQx at levels approaching human exposure. 8-COOH-MeIQx formation is catalyzed by P450 1A2 and appears to be a detoxication product. Thus, P450 1A2 is involved both in the metabolic activation and detoxication of this procarcinogen. 8-COOH- MeIQx is the principal metabolite of MeIQx excreted in human urine, but its formation has not been reported in other species (R. Turesky *et al.* Chem. Res. Toxicol. 11, 217-225). There are considerable interspecies differences in the metabolism of MeIQx, which effects the biological activity of this mutagen and must be considered when assessing human health risk.

#5308 INHIBITION OF ESTROGEN SULFOTRANSFERASE IN NORMAL HUMAN MAMMARY EPITHELIAL CELLS. Amy Lewis Nolan, Y. Otake, U. K Walle, and T. Walle, *Med Univ of South Carolina, Charleston, SC*

Cooked-food mutagens have recently been suggested to contribute to the etiology of breast cancer. 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), the most prevalent mutagen in the Western diet, is not carcinogenic until it is bioactivated by enzymes in the body. Previous observations have demonstrated that estrogen sulfotransferase (EST), present in normal human mammary epithelial (HME) cells, is a critical enzyme necessary for the bioactivation and subsequent DNA binding of N-hydroxylated PhIP (N-OH-PhIP) (*Carcinogenesis*, **19**: 2049-2053, 1998). Breast cancer initiation by this mechanism may be preventable. As naturally occurring phenols, present in our diet, have previously been shown to inhibit P-form phenol sulfotransferase (*Drug Metab. Dispos.*, **24**: 232–237, 1996), the objective of this study was to examine the inhibitory effect of two dietary phenols, quercetin and resveratrol, on EST activity. Both compounds potently inhibited recombinant EST in a competitive fashion with K_i values of 1 μM. To extend these studies to more physiologically relevant conditions the inhibition of EST was examined in intact cultured HME cells. The mean baseline EST activity in the HME cells was 4.4 pmol/hr/mg protein. The IC_{50} for resveratrol was very similar to that for recombinant EST, i.e. about 1 μM. Surprisingly, quercetin was ten times more potent in the HME cells with an IC_{50} of about 0.1 μM, a concentration that should be possible to achieve from the normal dietary content of this polyphenol. Supported by CA69138.

#5309 INVESTIGATION OF RESONANCE STABILIZATION EFFECTS IN THE BIOACTIVATION OF CARCINOGENIC AROMATIC AMINES. Jennifer C Sasaki, N. L Tran, M. E Colvin, and J. S Felton, *Lawrence Livermore National Lab, Livermore, CA*

Aromatic amines, found in diverse sources ranging from dyestuffs and cigarette smoke to cooked foods, are environmental pollutants with demonstrated mutagenic and carcinogenic effects. Aromatic amines require bioactivation before they can exert mutagenic effects. This activation rate is believed to be a key factor in determining the carcinogenic potency of these compounds. A majority of evidence indicates that a nitrenium ion or nitrenium-like N-esters are the ultimate DNA-reactive metabolites in the aromatic amine bioactivation pathway, although studies have also proposed the neutral nitrene species as a possible intermediate. To better understand aromatic amine bioactivation, classical resonance theory and *ab initio* quantum chemical calculations were employed to determine the relative stabilities of nitrene and nitrenium ion forms for a range of substituted aromatic amines. In general, it was determined that substitution of electron donating groups stabilized formation of the singlet nitrene and nitrenium ion, whereas electron withdrawing group substitution destabilized these systems. For the simple aromatic amine system aniline, resonance theory predictions of the effects of para-substituted electron donating and withdrawing groups agreed with the quantum chemical stabilization energies for the singlet states of the nitrene and nitrenium forms. For larger aromatic systems containing a 2-aminobiphenyl or 2-aminofluorene backbone, similar agreement was observed between resonance predictions and computational results, with variations arising from differences in the degree of conjugation between the linked phenyl rings. The results presented here provide validation for the use of resonance concepts to understand aromatic amine mutagenic and carcinogenic potency in biological systems. (Work carried out by LLNL under contract W-7405-ENG-48 from the U.S. DOE and supported by NCI grant CA55861.)

#5310 CARCINOGENESIS STUDIES BY VARIOUS ADMINISTRATIONS OF BENZENEDIAZONIUM SULFATE IN MICE. Bela Toth, and P. Gannett, *Nebraska Med Ctr, Omaha, NE, and West Virginia Univ, Morgantown, WV*

The goal of the investigations was to explore the full range of the carcinogenic action of benezenediazonium sulfate (BD) by using different dose levels and routes of administrations in Swiss mice. The chemical was given by subcutaneous and oral administrations. The subcutaneous administration consisted of three experiments: a. BD was given at 10 μg/g as 26 weekly injections; b. BD was given at 25 μg/g as 10 weekly injections; and c. BD was given at 50 μg/g as a single injection. In the oral administration BD was given at 100 μg/g as 52 weekly gavages. Each group consisted of 100 (50 ♀, 50 ♂) mice. In the subcutaneously treated mice, the incidences of tumors of subcutis were: a. 42% in females and 26% in males; b. 8% in females and 8% in males; and c. 2% in females and 4%